



TITLE:

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**No viral evolution in the lymph nodes of SIV-infected rhesus macaques during
combined antiretroviral therapy**

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Running title: No SIV evolution in lymph nodes during cART.

22

Abstract

23

24 To elucidate the mode of viral persistence in primate lentivirus-infected individuals during
25 combination antiretroviral therapy (cART), four simian immunodeficiency virus
26 (SIV)239-infected monkeys were treated with cART for 1 year. The viral *env* genes prepared
27 from total RNA extracted from the mesenteric lymph nodes by single genome amplification
28 were assessed at the completion of therapy. Analyses of nucleotide substitution and
29 phylogeny revealed no viral evolution during cART.

30

Combination anti-retroviral therapy (cART) has transformed human immunodeficiency virus (HIV) infection from incurable to a manageable disease. It suppresses the viral burden in patients to undetectable levels (14, 16, 27), lowers the chance of viral transmission (28), increases the number of CD4⁺ T lymphocytes (14, 16), reconstitutes immunity (4, 24, 38), and extends the life expectancy of patients (1). However, cART does not cure patients due to its inability to eradicate the virus from infected individuals (7), suggesting the existence of a viral reservoir that is refractory to cART. Its identification and eradication are, therefore, requisites for a functional cure for acquired immunodeficiency syndrome (AIDS). To establish a strategy for eradication of the HIV reservoir, the mechanism of persistence of the virus must be elucidated. Two mechanisms of viral persistence have been proposed: one is ongoing cycles of viral replication despite the presence of antivirals (37), and the other is provirus integration into long-lived cells (32). Whereas preceding studies concerning this issue have been extensively conducted employing clinical specimens from HIV-1-infected patients, including plasma, peripheral blood mononuclear cells, and gut-associated lymphatic tissues (2, 11, 23), lymph nodes, which are epicenters of virus replication in infected individuals not undergoing therapy (10, 25, 26), have been subjected to scrutiny only rarely. In animal models of cART, in particular the simian immunodeficiency virus (SIV)/macaque model, which allows for systemic examination, the identification of the viral reservoir and the mechanism of viral holding have not been studied in detail.

To elucidate how the virus is maintained during cART in an animal model of anti-HIV chemotherapy, we administered a combination of nucleotide/nucleoside reverse transcriptase inhibitors (azidothymidine [AZT], lamivudine [3TC], and tenofovir disoproxil

fumarate [TDF]) and protease inhibitors (lopinavir [LPV] with ritonavir [RTV]) to four SIV239-infected rhesus macaques for 1 year (18). Although the plasma viral RNA loads of the animals were suppressed to below the assay detection limit during the period of chemotherapy, systemic analysis conducted at the completion of the therapy revealed viral RNA present in lymphatic tissues, especially in mesenteric and splenic lymph nodes (MLN and SLN, respectively) at high titers. Reasoning that any possible mode(s) of viral persistence should be in operation in tissues with high levels of viral RNA expression, we investigated viral genes in these tissues.

It is expected that viral genes accumulate nucleotide substitutions in proportion to time post-infection in individuals not undergoing therapy due to continuous virus replication mediated by the error-prone viral reverse transcriptase. Such mutation rates have indeed been observed in the V3 loop of *env*, p17 of *gag* (20), and C2-C5 region of *env* (30) in HIV-1 infected patients as well as in the *env* gene from monkeys experimentally infected with SIV (5, 19). We hypothesized that viral genes would accumulate mutations if the virus was continuously replicating in the reservoir despite the presence of antivirals.

First, to ascertain whether such accumulation of mutations took place to a detectable magnitude in our experimental system, SIV239, a molecularly cloned virus, was used to infect macaques for 1 year, and we periodically sampled viral genes from the untreated control animal (MM521). To reveal ongoing expression of viral genes at sampling, total RNA was extracted from plasma samples collected at 8, 18, 42, and 68 weeks post-infection (wpi) and examined. Single genome amplification (SGA) (29) was used to amplify present viral genes and to avoid selective amplification of a particular genotype or recombination between genotypes during polymerase chain reaction (PCR). Using nested

PCR, we amplified the entire *env* gene, which accumulates nucleotide substitutions in the greatest numbers, following reverse transcription of cDNA from the extracted RNA. The initial cycles of PCR were carried out utilizing the following primers: forward, SIV20F (5'-ctc cag gac tag cat aaa tgg-3'); reverse, SHenv9R (5'-ggg tat cta aca tat gcc tc-3'). Successive PCR cycles were run with the following primers: forward, SIV21F (5'-ctc tct cag cta tac cgc cc-3'); reverse, SHenv8R (5'-gcc ttc ttc ctt ttc taa g-3'). The PCR products from an average of 12 independent reactions per time point were directly subjected to sequencing.

We computed the number of mutations in each SGA clone obtained from plasma samples of an untreated monkey (MM521) through a comparison with that of the inoculum virus (Fig. 1). A linear relationship with a coefficient of 1.25×10^{-4} ($r^2 = 0.8503$, $p < 0.0001$; GraphPad Prism, La Jolla, CA, USA) was revealed between the number of mutations in the SGA clones and the time post-infection. Using the coefficient, the cumulative mutations per annum was determined to be 6.5×10^{-3} substitutions/site/year, a comparable figure to those of SIV and HIV reported previously (9×10^{-3} (5, 19) and 6.0×10^{-3} (29) substitutions/site/year, respectively). The accuracy of the “molecular clock” in our experimental setting prompted us to examine viral RNA extracted from the lymph nodes of animals that underwent cART for 1 year.

Total RNA was extracted from the MLN of four treated animals and one untreated animal as well as the SLN of one of the treated animals (MM530) at the completion of the observation period and used as template for PCR; the products were subjected to sequence analysis as described above. On average, 10 sequences were obtained from each sample (Fig. 2A and Table). The number of mutations observed in the *env* gene from MM521 (untreated) was, on average, 25 of 2700 bases. In contrast, the number in treated animals was on average

1.5 of 2700 bases (Table). The difference in the number of mutations in *env* between the plasma and MLN samples at 68 wpi (at necropsy) in the untreated animal, MM521, was statistically insignificant ($p > 0.05$; Fig. 2A), justifying our comparison of these two distinct anatomical compartments. Thus, we proceeded to compare the substitution numbers in the plasma at 8 wpi, immediately before the onset of cART, with those from the lymph nodes of animals treated with cART at necropsy (61-65 wpi). Nucleotide substitutions in the *env* gene in both plasma and the MLN of the untreated animal (MM521) at 68 wpi increased compared to that in plasma at 8 wpi ($p < 0.0001$). In contrast, those in the MLN of treated animals at the completion of cART were unchanged (MM528 and SLN of MM530) or decreased significantly (MM491, MM499, and MLN of MM530) (Fig. 2A). The results indicated that virus did not accumulate further mutations beyond those obtained by 8 wpi.

As the samples were collected from animals at various time points post-infection, the numbers depicted in Fig. 2A were converted to substitutions/site/year (Fig. 2B) for further analysis. Comparison of the number of viral mutations in plasma at 8 wpi (median, 5.9×10^{-3} substitutions/site/year) with that in the MLN (median, 7.2×10^{-3} substitutions/site/year) in the untreated animal, MM521, indicated no statistical difference ($p = 0.6265$), as predicted by the analysis in Fig. 2A. Next, we compared the numbers in animals that underwent chemotherapy. At 8 wpi, the treated animals were equivalent to MM521 (an untreated animal) in terms of therapeutic status, since cART was started after sample collection at 8 wpi. Not unexpectedly, there was no statistically significant difference in plasma number between the untreated and treated animals (MM491, 8.5×10^{-3} ; MM499, 9.8×10^{-3} ; MM528, 1.6×10^{-2} ; MM530, 8.5×10^{-3} ; and MM521, 5.9×10^{-3} substitutions/site/year), except for MM528 ($p = 0.0048$ compared to those from untreated animal). In contrast, the

123 mutations per annum in the lymph nodes of treated animals collected at necropsy (median,
124 3.4×10^{-4} substitutions/site/year) were significantly lower than those in the plasma of the
125 animals at 8 wpi (median, 9.8×10^{-3} substitutions/site/year) ($p < 0.0001$). The number of
126 mutations per year in lymph nodes between untreated and treated macaques was also
127 significantly different ($p < 0.0001$). This supports the hypothesis that ongoing viral
128 replication contributed little, if anything, to viral persistence during cART.

129 Examination of the nucleotide substitution numbers did not indicate discernible *de*
130 *novo* virus replication during cART. Therefore, we next investigated continuous viral
131 replication during cART through phylogenetic analysis of viral *env* clones. Clones were
132 obtained from the untreated animal (derived from plasma at 8, 18, 42, and 68 wpi and from
133 MLN) and from one of the treated animals (derived from plasma at 8 wpi and MLN at
134 necropsy) (Figs. 3 and S). To illustrate the accumulation and specific sites of mutations,
135 Highlighter plot analysis (<www.hiv.lanl.gov>) was also performed. Phylogenetic analysis of the
136 viral genes from the untreated animal revealed that i) *env* clones from plasma exhibited
137 increasing genetic distance from the inoculum virus with time, ii) clones obtained at a given
138 time point branched out of the one immediately before, a clear demonstration of viral
139 evolution, and iii) clones from the lymph node formed a cluster with those from plasma
140 collected at the same time. In contrast, clones from treated animals, regardless of the tissue
141 origin or time point, formed a cluster with clones derived from plasma of the untreated
142 animal at 8 wpi and the inoculum virus (Figs. 3 and S). The results of the Highlighter plot
143 analysis were consistent with those of the phylogenetic analysis. These results clearly
144 demonstrated that viral evolution did not take place in SIV239-infected rhesus macaques
145 during cART. Analysis of the *env* genes in the peripheral blood mononuclear cells and

gut-associated lymphatic tissues obtained from HIV-1 patients in cART also found no evidence of *de novo* viral replication (11).

In contrast, other studies have reported continuous virus replication during combined chemotherapy (2, 37). One possible explanation of this discrepancy is the thorough suppression of the plasma viral burden, < 20 copies/mL at necropsy that was achieved in this study (18). *De novo* virus replication was detected in HIV-1 patients whose plasma viral RNA burdens ranged from 20 to 400 copies/mL, but not in those with less than 20 copies/mL (15). Our findings also indicate that the cART regimen we employed (18) was robust enough to halt viral evolution nearly completely in animals.

Our sample size, an average of 10 sequences from each specimen, conceivably limited our ability to detect minor populations with signs of ongoing replication. An analysis of four animals, however, did not reveal the genotypes detailed in the current study. Therefore, while our results cannot rule out possible *de novo* viral replication during cART, the data indicate that it is not a major mode of viral persistence in individuals whose virus replication levels are thoroughly suppressed by cART.

The locations of other potential viral reservoirs, in addition to resting CD4⁺ T lymphocytes, an already established HIV/SIV reservoir found to be present in blood (6, 8, 9, 13), lymph nodes (6), and the spleen (31), remains elusive. While the cART regimen we developed suppressed viral RNA levels nearly completely in the circulation and fairly well in effector sites, such as the gastrointestinal tract and lung, viral RNA expression levels in lymph nodes were not contained effectively (18), suggesting that the viral reservoir consists of cells present in lymph nodes. We also detected CD3-positive cells, most likely CD4⁺ T lymphocytes, expressing Nef protein in the follicles of the MLN of an SIV-infected animal

that exhibited viral rebound upon cessation of cART (18). Based on their location, these might be Tfh cells, which are of the memory phenotype (21, 22, 36). The results of the current study have further narrowed the location of the viral reservoir from our previous study (18) to cells with longer half-lives, which retain provirus for at least 1 year. Since resting CD4⁺ T cells possess long half-lives (33), these cells satisfy this criterion for a viral reservoir during cART. It is conceivable that resting CD4⁺ T cells functioned as the predominant viral reservoir in the SIV239/rhesus macaque model for patients on cART employed in our study, as in preceding studies concerning the issue in the context of HIV and SIV infections.

Lymph nodes serve as a major HIV reservoir throughout the course of infection without intervention by cART (10, 25, 26). During clinical latency, the virus persists as an intact provirus, which can produce infectious viral particles upon cell activation, in a miniscule fraction of the resting CD4⁺ T lymphocytes in lymph nodes (6). An extensive examination of lymph node specimens from HIV patients on cART revealed an infinitesimal amount of viral RNA-positive cells by *in situ* hybridization (17). Hockett *et al.* (17) revealed that cART lowers the number of viral RNA-positive cells in lymph nodes but that the number of viral copies in each infected cell is constant, regardless of the viral burden in the circulation, suggesting the existence of virus-infected cells actively transcribing viral genes under cART, as we found previously in the lymph nodes of SIV239-infected animals on cART (18). Taking our current observations together with those of Hockett *et al.* (17), the viral RNA-positive cells present in lymph nodes during cART may represent cells infected with virus prior to the initiation of cART and transcribing viral RNA from integrated provirus during therapy.

192 Current cART is unable to eradicate the viral reservoir; or more precisely, provirus
193 integrated in the reservoir. Based on our results, it is important to establish strategies to target
194 specifically long-lived cells that harbor intact provirus while unlocking the dormant state of
195 the provirus, perhaps using histone deacetylase (3), to achieve a functional cure for AIDS.
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Table. Origins and numbers of *env* clones.

ANIMAL ID	cART*	SPECIMEN	SAMPLE COLLECTION (WPI)	No. SGA** CLONES	No. NUCLEOTIDE SUBSTITUTIONS	
					MINIMUM/MAXIMUM	MEAN \pm SD
MM521	UNTREATED	PLASMA	8	10	1 / 8	3.3 \pm 2.2
		PLASMA	68	10	20 / 29	24.9 \pm 3.2
		MLN [#]	68	12	20 / 33	25.0 \pm 3.4
MM491	TREATED	PLASMA	8	10	1 / 7	3.5 \pm 1.8
		MLN [#]	63	10	0 / 2	0.6 \pm 0.8
MM499	TREATED	PLASMA	8	11	2 / 7	4.2 \pm 1.7
		MLN [#]	64	10	0 / 4	0.7 \pm 1.3
MM528	TREATED	PLASMA	8	10	4 / 11	6.6 \pm 2.4
		MLN [#]	61	11	0 / 3 (12 [†])	1.7 \pm 1.1 ^{††}
MM530	TREATED	PLASMA	8	10	2 / 8	4.0 \pm 1.7
		MLN [#]	65	10	0 / 4	2.1 \pm 1.2
		SLN ^{##}	65	10	0 / 6	2.4 \pm 1.9

*Combination anti-retroviral therapy; **single genome amplification; [#]mesenteric lymph node; ^{##}splenic lymph node; [†]interpreted as a hypermutant driven by APOBEC3G/F (Hypermur 2.0, <www.hiv.lanl.gov>); ^{††}computed excluding the clone with hypermutation.

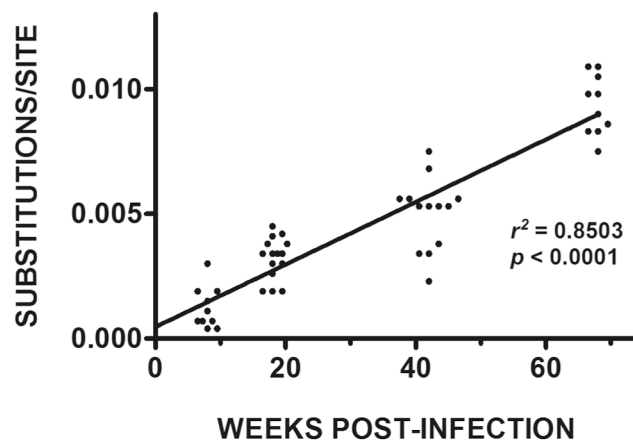


Fig. 1.

Time-dependent accumulation of nucleotide substitutions in SIV genomes circulating in an infected and untreated rhesus macaque. The sequences of viral *env* genes in circulation collected at 8, 18, 42, and 68 wpi from SIV239-infected animals and an untreated animal (MM521) were determined. Tamura-Nei distances (34) of the sequences were computed with the MEGA5 software (35), and the number of nucleotide substitutions per site were plotted against the number of wpi. Each symbol represents a single genomic amplicon derived from plasma samples collected at the time points designated above.

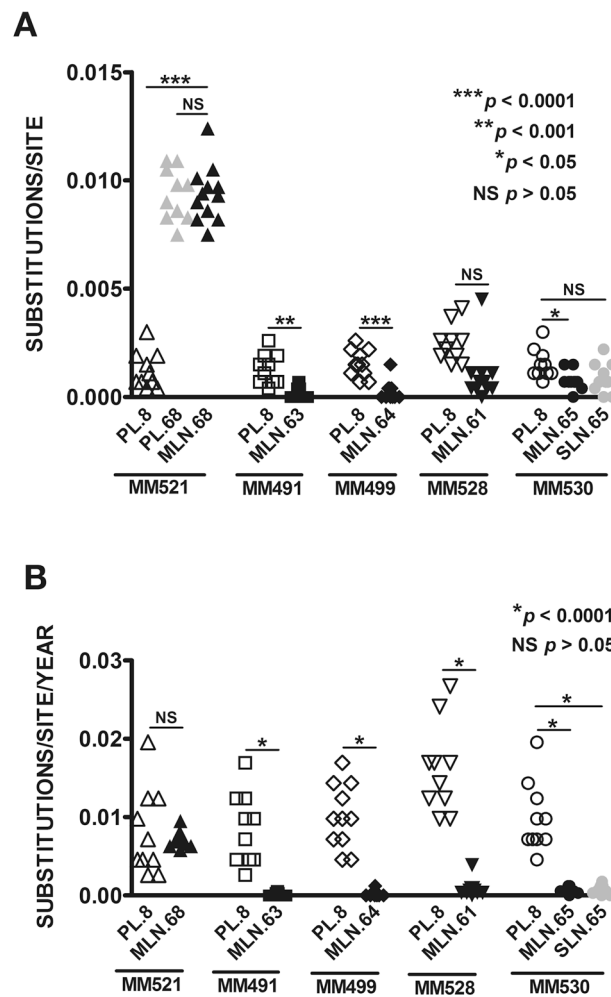


Fig. 2.

Nucleotide substitutions in *env* genes from SIV239-infected animals. The number of mutations in *env* from the plasma (PL, at 8 and 68 wpi) and mesenteric lymph node (MLN, at 68 wpi) of an SIV-infected but untreated animal (MM521) and from the plasma (at 8 wpi) and MLN (at necropsy, 61, 63, 64, or 65 wpi) plus splenic lymph node (SLN, at necropsy, 65 wpi) from SIV-infected and treated monkeys (MM491, MM499, MM528, and MM530) were assessed as described in the legend to Fig. 1. (A) Nucleotide substitutions per site are shown. The statistical significance of differences between substitution numbers was evaluated by Student's *t*-test using GraphPad Prism. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$; NS, $p > 0.05$. (B) Nucleotide substitutions per annum are shown. * $p < 0.001$; ** $p > 0.05$.

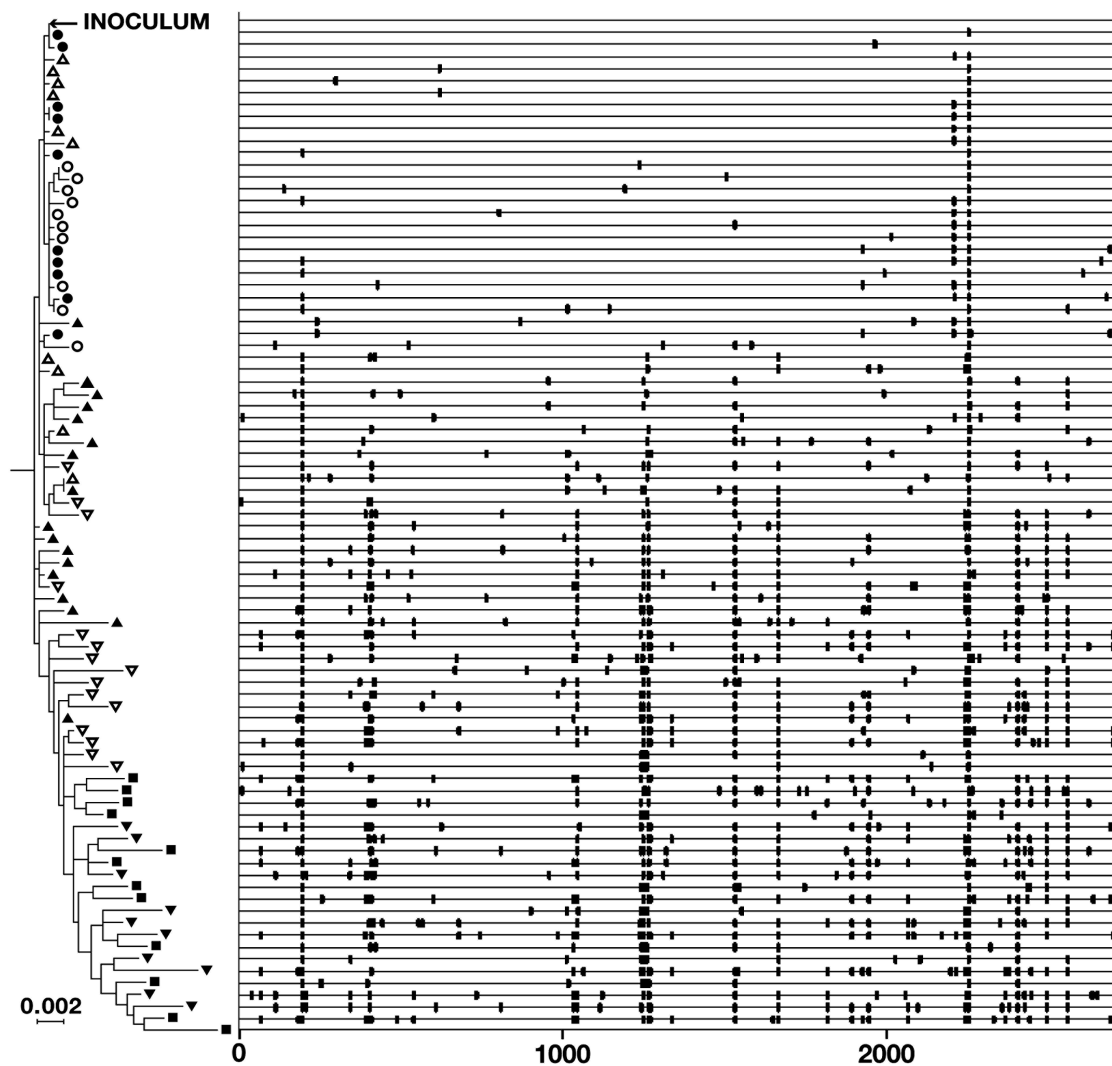
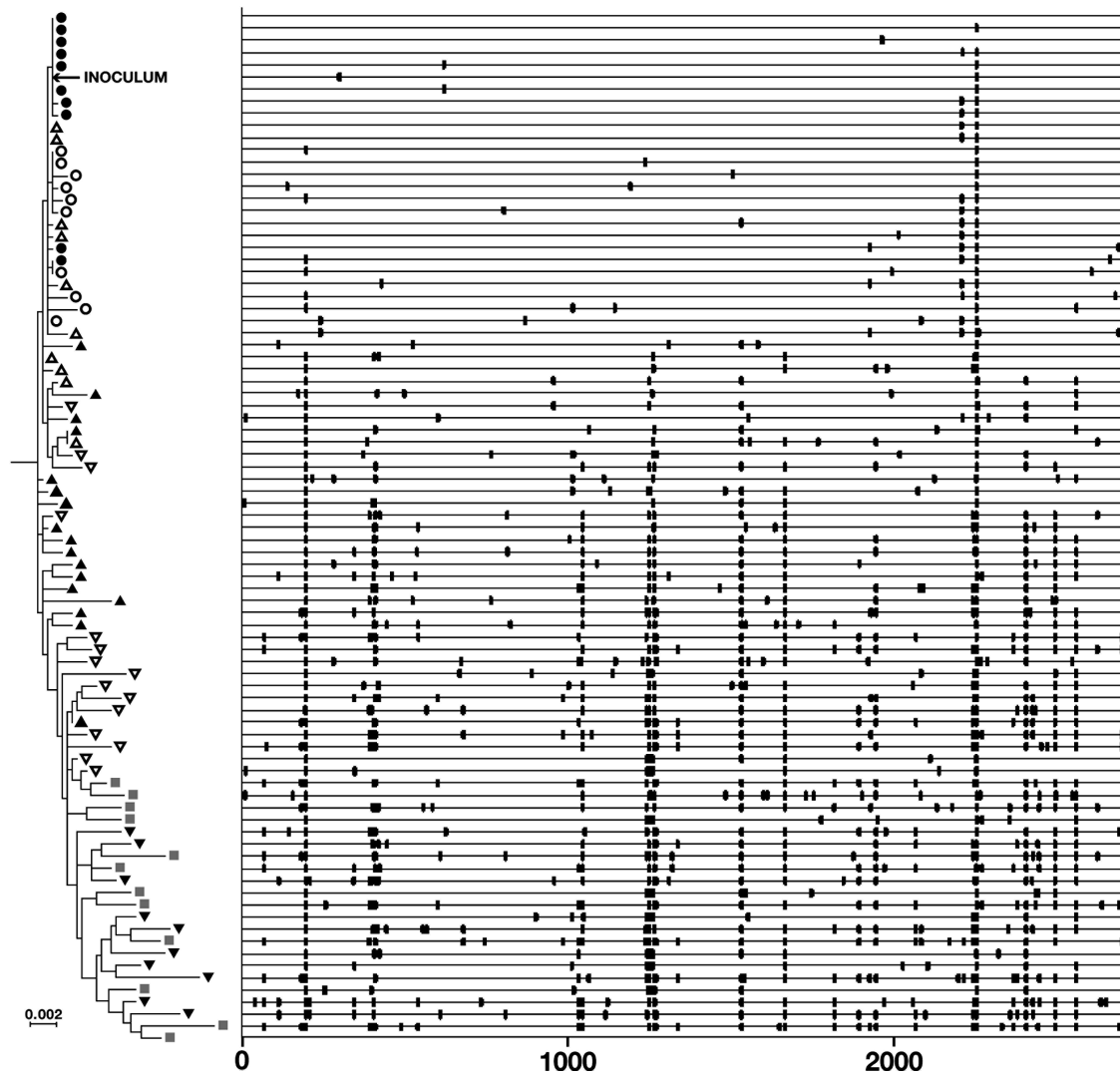
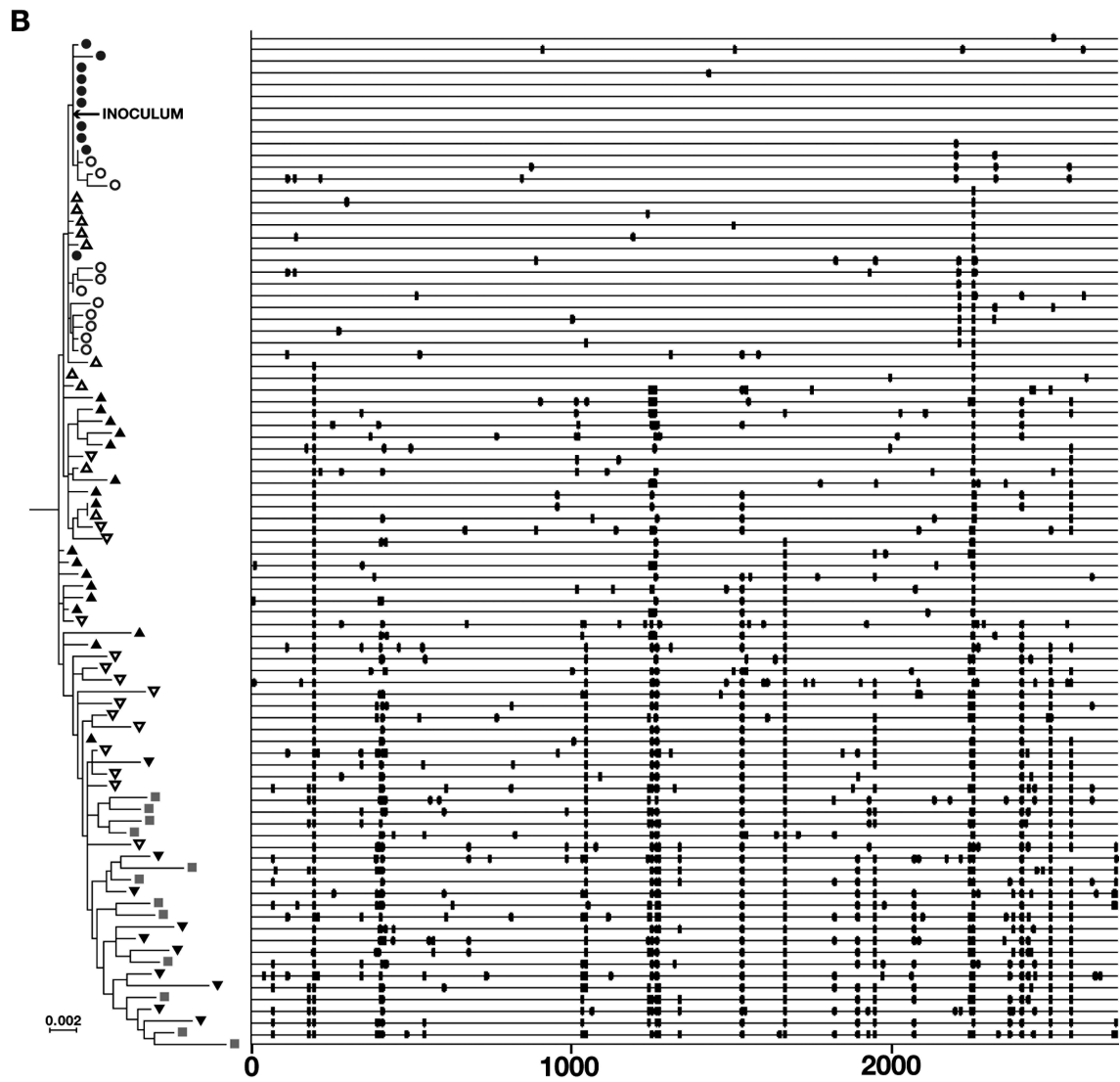


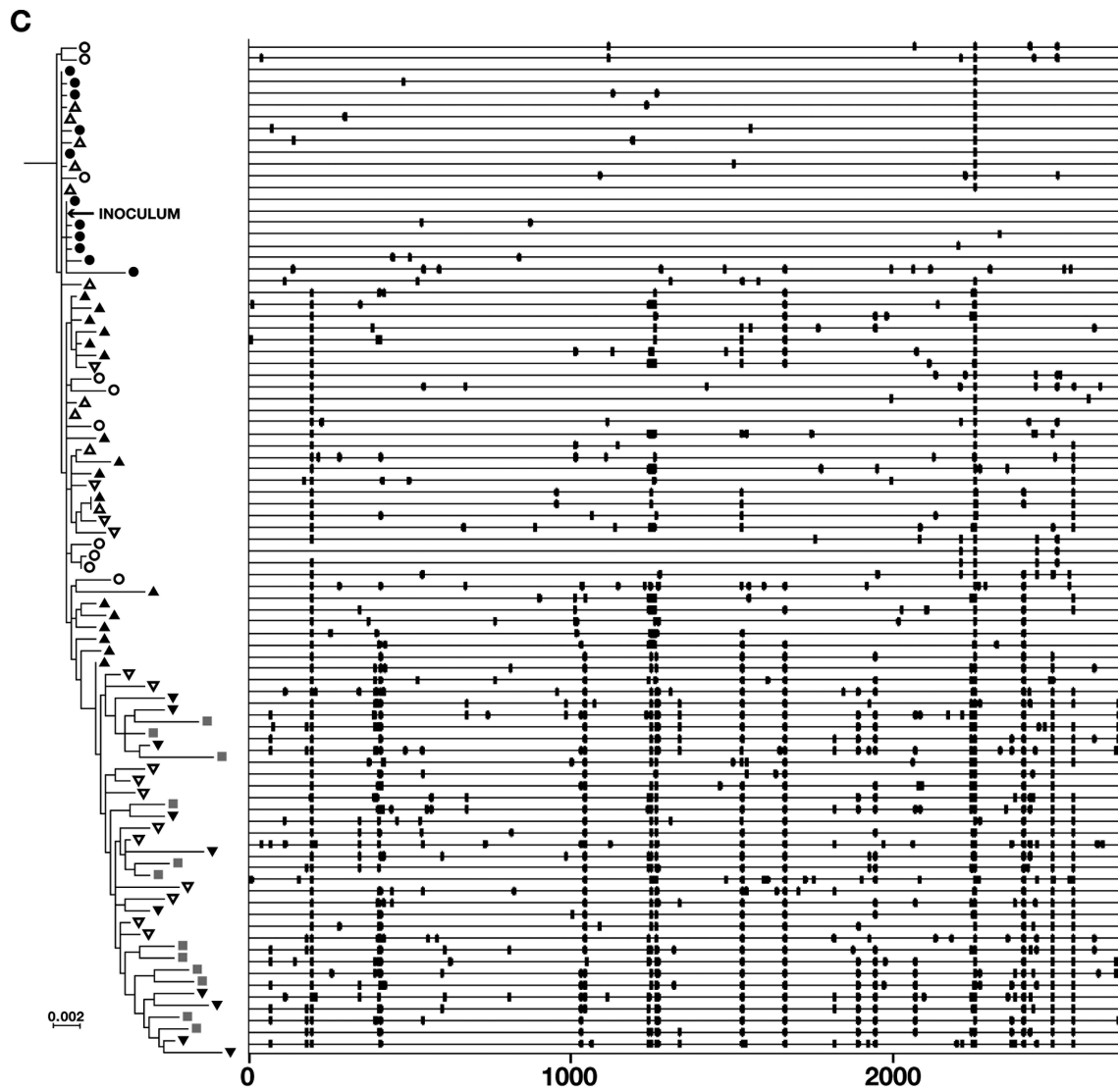
Fig. 3.

Phylogenetic relationship of *env* sequences from treated (MM530) and untreated (MM521) SIV-infected animals. Sequences of the entire *env* gene from both animals were subjected to phylogenetic analysis. Phylogenetic trees were constructed using the Maximum Likelihood method (12). Open circles, sequences in the plasma of MM530 at 8 wpi; closed circles, those from the MLN of MM530 at 65 wpi; open triangles, those from plasma of MM521 at 8 wpi; closed triangles, those from plasma of MM521 at 18 wpi; open inverse triangles, those from plasma of MM521 at 42 wpi; closed inverse triangles, those from plasma of MM521 at 68 wpi; closed rectangles, those from MLN of MM521 at 68 wpi. The scale represents a genetic distance equivalent to 0.002 substitutions per site. The corresponding sequence of SIVmac251 32H (GenBank accession no. D01065) was applied as an outgroup.

A







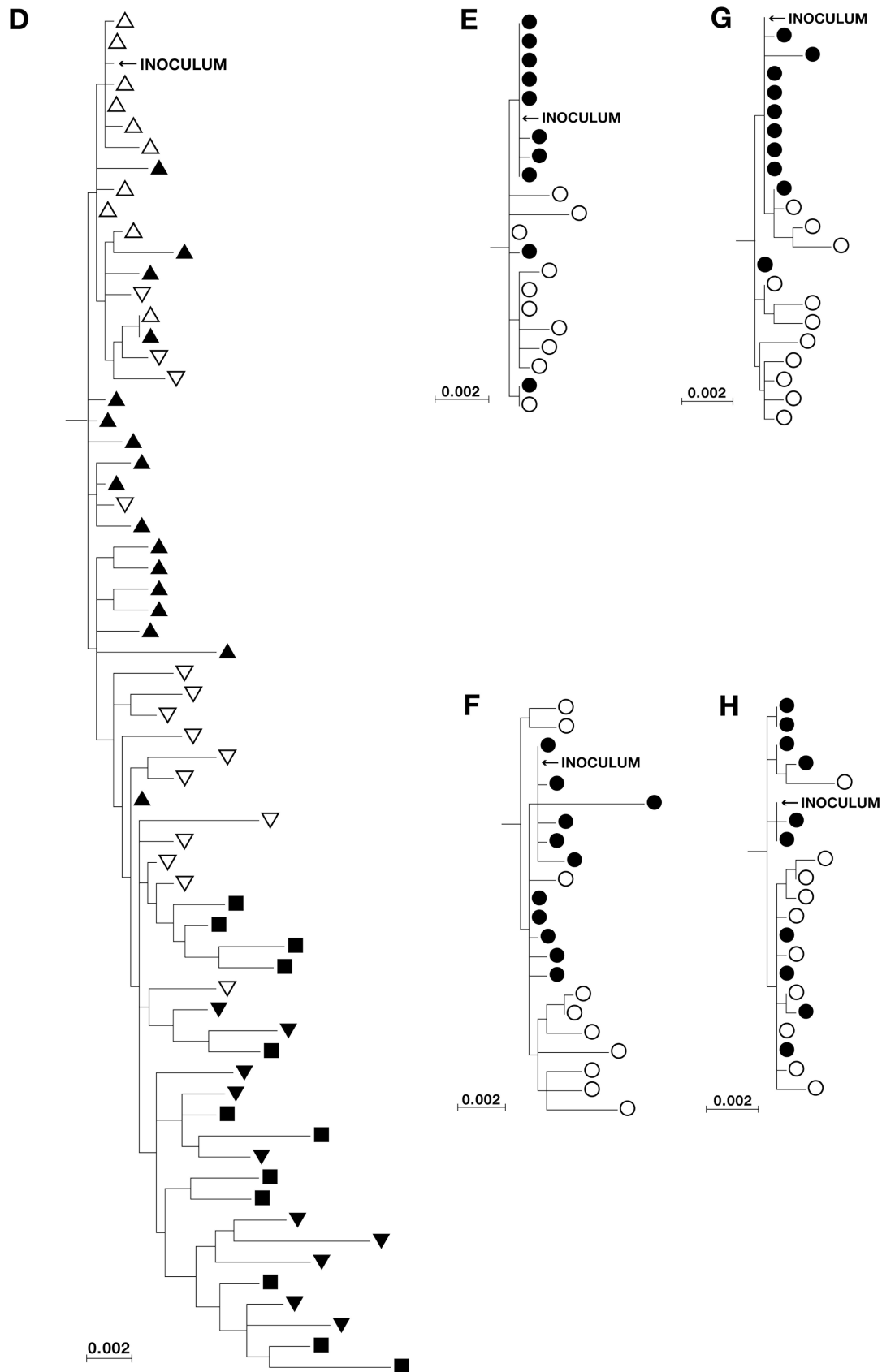


Fig. S.

Phylogenetic relationships of *env* sequences from treated and untreated animals. Sequences were subjected to phylogenetic analysis as described in the legend to Fig. 3. Sequences from MM521 (untreated) and (A) MM491 (treated), (B) MM499 (treated), and (C) MM528 (treated) are shown. Phylogenetic trees generated using the sequences from an individual animal are also shown (D, MM521; E, MM491; F, MM499; G, MM528; and H, MM530). Open circles, sequences in the plasma of treated animals at 8 wpi; closed circles, those from MLN of treated animals at the completion of cART; open triangles, those from plasma of MM521 at 8 wpi; closed triangles, those from plasma of MM521 at 18 wpi; open inverse triangles, those from plasma of MM521 at 42 wpi; closed inverse triangles, those from plasma of MM521 at 68 wpi; closed rectangles, those from MLN of MM521 at 68 wpi. The scale represents a genetic distance equivalent to 0.002 substitutions per site. The corresponding sequence of SIVmac251 32H (GenBank accession no. D01065) was employed as an outgroup.